IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application Number	: 10/764,451	Confirmation No.:	8691
Applicants	: Craig A. TOWNSEND, *et al.*		
Filed	: January 27, 2004		
Title	: ANTIMICROBIAL COMPOUNDS		
TC/Art Unit	: 1639		
Examiner:	: J. Epperson		
Docket No.	: 62732.001152		
Customer No.	: **21967**		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

Sir,

I, James Dick, Ph.D., declare that:

1.) I am the Director of Bacteriology, Johns Hopkins Medical Institutions. I have received a Bachelor of Sciences degree in Biology from the Clemson University, Clemson, S,C. USA, in 1968 and a Doctor of Philosophy degree in Pathology from the University of Maryland, Baltimore, MD., USA, in 1983.

2.) I have been involved with teaching and research in the field of molecular biology and pathology for approximately 35 years, and am an author of approximately 70 publications in these fields. A recitation of some of these publications, together with details of my education, are given in my *curriculum vitae* which is attached as Exhibit A.

3.) I am a named inventor of U.S. Patent Application Serial Number 10/764,451 (hereinafter "the '451 application").

4.) This declaration is being filed to submit evidence in response to the Office Action mailed February 24, 2006 in the '451 application.

5.) I have read and am familiar with the specification and pending claims of the '451 application and the Final Office Action dated February 24, 2006.

6.) As a co-author, I am familiar with the following article:

- Parrish, N., Houston, T., Jones, P., Townsend, C., Dick, J., "In Vitro Activity of a Novel Antimycobacterial Compound, *N*-Octanesulfonylacetamide, and Its Effects on Lipid and Mycolic Acid Synthesis" *Antimicrobial Agents and Chemotherapy*, Apr. 2001, v. 45, pp. 1143-1150. ("the Parrish reference")

7.) The Parrish reference discloses the treatment of both pathogenic and non-pathogenic mycobacteria with *N*-octanesulfonylacetamide ("OSA"). *See* Parrish reference at 1145. Table 1 of the Parrish reference shows that OSA exhibits activity against the pathogenic bacteria, *M. bovis, M. bovis* BCG, *M. kansasii, M. avium complex,* and *M. paratuberculosis*, but not against the rapid growing non-pathogenic bacteria, *M. smegmatis, M. fortuitum, M. chelonei,* and *M. abscessus.*

8.) Because of the structural similarities between OSA and the compounds of formula I of the invention, these compounds would be expected to exhibit selective activity against slow-growning pathogenic mycobacteria.

7.) The undersigned acknowledges that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. 1001), and may jeopardize the validity of the application or any patent issuing thereon. The undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true.

I declare under penalty of perjury that the foregoing is true and correct.

Executed on August 23, 2006

Declarant's Signature: ______________________

Home Address: 15794 Dover Road
Upperco, MD 21155, USA

Exhibit A

James D. Dick **May 2006**

DEMOGRAPHIC INFORMATION

Current Appointments:
Associate Professor of Pathology, School of Medicine, Johns Hopkins University
Joint Appointments in Molecular Microbiology & Immunology.
School of Public Health, Johns Hopkins University
Associate Director, Medical Microbiology Division, Department of Pathology
Director of Bacteriology, Johns Hopkins Hospital

Personal Data:
Medical Microbiology Division
Department of Pathology
Meyer B1-193
The Johns Hopkins Medical Institutions
600 N. Wolfe Street
Baltimore, MD 21287-7093
(410) 955-5077 (phone)
(410) 614-8087 (fax)
jdick@jhmi.edu

Education and Training

B.S.	- 1968	Clemson University	Biology
M.S.	- 1970	Clemson University	Microbiology
Ph.D.	- 1983	University of Maryland School of Medicine	Experimental Pathology

Professional Experience

Captain, U.S. Army	Brooke Army Medical Center	1969-1970
Microbiology Technologist to Asst. Chief	Johns Hopkins Hospital	1970-1973
Chief/Laboratory Manager	Johns Hopkins Hospital	1973-1983
Assistant Professor, Pathology	JHU School of Medicine	1983-1992
Joint Appointment in Molecular Microbiology & Immunology	JHU School of Public Health	1986- Present
Director, Biochemical Microbiology	Johns Hopkins Hospital	1984-
Associate Professor, Pathology	JHU School of Medicine	1993- Present
Associate Director, Microbiology Division	JHU School of Medicine	1988- Present
Director of Bacteriology	Department of Pathology JHU School of Medicine	1994- Present

RESEARCH ACTIVITIES

PUBLICATIONS

PEER-REVIEWED SCIENTIFIC ARTICLES

1. Dick, J.D., W.G. Merz, and R. Saral. 1980. Incidence of polyene-resistant yeasts recovered from clinical specimens. Antimicrob. Agents Chemother. 18: 158-163.

2. Wingard, J.R., J.D. Dick, W.G. Merz, G.R. Sandford, R. Saral, and W.H. Burns. 1980. Pathogenicity of *Candida tropicalis* and *Candida albicans* after gastrointestinal inoculation in mice. Infect. Immun. 29: 808-813.

3. Wingard, J.R., J.D. Dick, W.G. Merz, G.R. Sandford, and W.G. Burns. 1982. Differences in virulence of clinical isolates of *Candida tropicalis* and *Candida albicans* in mice. Infect. Immun. 37: 833-836.

4. Markman, J., J. Manisi, J.D. Dick, B. Filburn, G.W. Santos, and R. Saral. 1982. Trimethoprim-sulfamethoxazole resistant *Streptococcus pneumoniae* causing sepsis in a bone marrow transplant recipient with chronic graph-versus-host disease. J.A.M.A. 248: 3011-3012.

5. Filburn, B., V. Shull, Y. Tempera, and J. Dick. 1983. Evaluation of an automated fluorescence polarization immunoassay for vancomycin. Antimicrob. Agents Chemother. 24: 216-220.

6. Dick, J.D., B.R. Rosengard, W.G. Merz, R.K. Stuart, G.M. Hutchins, and R. Saral. 1985. Fatal disseminated candidiasis due to amphotericin B resistant *Candida guilliermondii*. Ann. Intern. Med. 102: 67-68.

7. Green, L., J.D. Dick, S.P. Goldberger, and C.M. Angelopulos. 1985. Prolonged elimination of piperacillin in a patient with renal and liver failure. Drug Intelligence Clin. Pharm. 19: 427-429.

8. Shull, V. and J.D. Dick. 1985. Determination of ticarcillin levels in serum by high-pressure liquid chromatography. Antimicrob. Agents Chemother. 28: 597-600.

9. Karp, J.E., J.D. Dick, C. Angelopulos, P. Charache, L. Green, P.J. Burke, and R. Saral. 1986 Empiric use of vancomycin during prolonged treatment-induced granulocytopenia: A randomized, double-blind, placebo-controlled clinical trial in patients with acute leukemia. Amer. J. Med. 81: 237-242.

10. Morton, S.J., V.H. Shull, and J.D. Dick. 1986. Determination of norfloxacin and ciprofloxacin concentrations in serum and urine by high-pressure liquid chromatography. Antimicrob. Agents Chemother. 30: 325-327.

11. Wingard, J.R., J.D. Dick, P. Charache, and R. Saral. 1986. Antibiotic-resistant bacteria in surveillance stool cultures of patients with prolonged neutropenia. Antimicrob. Agents Chemother. 30: 435-439.

12. Hamilton, S.R., J. Hyland, D. McAvinchey, Y. Chandhoy, L. Hartka, H.T. Kim, P. Cichen, J. Floyd, N. Turjman, G. Kessie, P.O. Nair, and J.D. Dick. 1987. Effects of chronic dietary beer and ethanol consumption on experimental colonic carcinogenesis by azoxymethane in rats. Cancer Res. 47: 1551-1559.

13. Dick, J.D., V. Shull, J.E. Karp, and J. Valentine. 1988. Bacterial and host factors effecting *Pseudomonas aeruginosa* colonization versus bacteremia in granulocytopenic patients. Eur. J. Cancer Clin. Oncology. 24, Suppl, S47-S54.

14. Karp, J.E., J.D. Dick, and W.G. Merz. 1988. Systemic infection and colonization with and without prophylactic norfloxacin use over time in the granulocytopenic, acute leukemia patient. Eur. J. Cancer Clin. Oncology. 24, Suppl 1, S5-S13.

15. Rowley, S.D., J. Davis, J.D. Dick, H.G. Brain, P. Charache, R. Saral, L.L. Sensenbrenner, and G.W. Santos. 1988. Bacterial contamination of bone marrow grafts intended for autologous and allogeneic bone marrow transplantation: Incidence and clinical significance. Transfusion, 28: 109-112.

16. Sawusch, M.R., T.P. O'Brien, J. Valentine, J.D. Dick, and J.D. Gottsch. 1988. Topical imipenem therapy of aminoglycoside - resistant *Pseudomonas* keratitis in rabbits. Am. J. Ophthalmol. 106: 77-81.

17. Sawusch, M.R., T.P. O'Brien, J.D. Dick, and J.D. Gottsch. 1988. Use of collagen corneal shields in the treatment of bacterial keratitis. Am. J. Ophthalmol. 106: 279-281.

18. Holroyd, K.J., A.P. Reiner, and J.D. Dick. 1988. *Streptobacillus moniliformis* polyarthritis mimicking rheumatoid arthritis. An urban case of rat bite fever. Amer. J. Med. 85: 711-714.

19. O'Brien, T.P., M.R. Sawusch, J.D. Dick, T.R. Hamburg, and J.D. Gottsch. 1988. Use of collagen corneal shields versus soft contact lenses to enhance penetration of topical tobramycin. J. Cataract Refract. Surg. 14: 505-507.

20. O'Brien, T.P., M.R. Sawusch, J.D. Dick, and J.D. Gottsch. 1988. Topical ciprofloxacin treatment of *Pseudomonas* keratitis in rabbits. Arch. Ophthalmol. 106: 1444-1446.

21. Aguayo, J.B., M.P. Gamcsik, and J.D. Dick. 1988. High resolution deuterium NMR studies of bacterial metabolism. J. Biol. Chem. 263: 19552-19557.

22. Dumler, J.S., G.J. Osterhout, J.G. Spangler, and J.D. Dick. 1989. *Vibrio cholerae* serogroup non-01 cystitis. J. Clin. Microbiol. 27: 1898-1899.

23. Hantel, A., J.D. Dick, and J.E. Karp. 1989. Listeriosis in the setting of malignant disease: changing issues in an unusual infection. Cancer 64: 516-520.

24. Karp, J.E., W.G. Merz, J.D. Dick, R. Saral, and P.J. Burke. 1990. Management of infectious complications of acute leukemia and antileukemia therapy. Oncology 4: 45-53.

25. Cantu, T.G., J.D. Dick, D.E. Elliott, R.L. Humphrey, and D.M. Kornhauser. 1990. Protein binding of vancomycin in an IgA myeloma patient. Antimicrob. Agents Chemother. 34:1459-1461.

26. von Graevenitz, A., G. Osterhout, and J.D. Dick. 1991. Grouping of some clinically relevant Gram-positive rods by automated fatty acid analysis: Diagnostic implications. Arch. Microbiol. Path. Infect. Scand. 99:147-154.

27. Gottsch, J.D., M.L. Gilbert, D.F. Goodman, M.E. Salewski, J.D. Dick, and W.J. Stark. 1991. Excimer laser ablative treatment of microbial keratitis. Ophthalmology 98:146-149.

28. Valentine, J.L., R.R. Arthur, H.L.T. Mobley, and J.D. Dick. 1991. Detection of *Helicobacter pylori* using the polymerase chain reaction. J. Clin. Microbiol. 29:689-695.

29. Osterhout, G.J., V.H. Shull, and J.D. Dick. 1991. Identification of clinical isolates of gram negative non-fermentative bacteria by an automated cellular fatty acid identification system. J. Clin. Microbiol. 29:1822-1830.

30. Morrow, J.F., H.G. Braine, T.S. Kickler, P.M. Ness, J.D. Dick, and A.K. Fuller. 1991. Septic reactions to platelet transfusions: A persistent problem. JAMA. 226:555-558.

31. Karp, J.E., W.G. Merz, J.D. Dick, and R. Saral. 1991. Strategies to prevent or control infections after bone marrow transplants. Bone Marrow Transplantation. 8:1-6.

32. Goepp, J.G., C.K.K. Lee, T. Anderson, J.D. Dick, J.M. Stokoe, and J. Eiden. 1992. Use of ciprofloxacin in an infant with ventriculitis. J. Pediatrics 91:412-415.

33. Karp, J.E., W.G. Merz, and J.D. Dick. 1993. Management of infections in neutropenic patients: advances in therapy and prevention. Curr. Opin. Infect. Dis. 6:405-411.

34. Butz, A.M., P. Fosarelli, J. Dick, T. Cusack, R. Yolken. 1993. Prevalence of Rotavirus on high risk fomites in day care facilities. Pediatrics. 92:202-205

35. Cherian, T., M.C. Steinhoff, L.H. Harrison, D. Rohn, L.K. McDougal, and J.D. Dick. 1994. A cluster of invasive pneumococcal disease in young children in child care. JAMA. 271:695-697.

36. Karp, J.E., W.G. Merz, and J.D. Dick. 1994. Management of infections in neutropenic patients: New opportunities and emerging challenges. Curr. Opin. Infect. Dis. 7:430-435.

37. Kuhajda, F.P, K. Jenner, F.D. Wood, R.A. Hennigar, L.B. Jacobs, J.D. Dick, and G.R. Pasternack. 1994. Fatty acid synthesis: A potential selective target for antineoplastic therapy. Proc. Natl. Acad. Sci. USA 91:6379-6383.

38. Curtis, M.J., P.R. Brown, J.D. Dick, and R.H. Jinnah. 1995. Contaminated fractures of the tibia in a comparison of treatment modalities in an animal model. J. Orthop. Res. 13:286-295.

39. Aguilar, H.E., T.A. Meredith, A. Shaarawy, M. Kincaid, and J.D. Dick. 1995. Vitreous cavity penetration of ceftazidine after intravenous administration. Retina. 15:154-159.

40. Shaarawy, A., T.A. Meredith, M. Kincaid, J.D. Dick, E. Aguilar, D.J. Richie, and R.M. Reichley. 1995. Intraocular injection of ceftazidime: Effects of inflammation and surgery. Retina 15:433-438.

41. Meredith, T.A., H.E. Aguilar, A. Shaarawy, M. Kincaid, and J.D. Dick. 1995. Vancomycin levels in the vitreous cavity after intravenous administration. Am. J. Opthalmol. 119:774-778.

42. Aguilar, H.E., T.A. Meredith, A. El-Massry, A. Shaarawy, M. Kincaid, J.D. Dick, D.J. Ritchie, R.M. Reichley, and M.K. Neisman, 1995. Vancomycin levels after intravitreal injection: Effects of inflammation and surgery. Retina 15:428-432.

43. El-Massry, A., T.A. Meredith, H.E. Aguilar, A. Shaarawy, M. Kincaid, J. Dick, M.I.E. Mahmoud. 1996. Aminoglycoside levels in rabbit vitreous cavity after intravenous administration. Am. J. Ophthalmology. 122:684-689.

44. Sicherer, S.H., Asturias, J.A. Winkelstein, J.D. Dick and R.E. Willoughby. 1997. *Francisella philomiragia* sepsis in chronic granulomatous disease. Pediatric Infect. Dis. J. 16: 420-422.

45. Osterhout, G., J.L. Valentine, and J.D. Dick. 1998. Phenotypic and Genotypic characterization of clinical strains of CDC Group IV c-2. J. Clin Microbiol. 36: 2618-2622.

46. Parrish, N.M., J.D. Dick, and W.R. Bishai. 1998. Mechanisms of latency in Mycobacterium tuberculosis. Trends in Microbiology. 6:107-112.

47. Stoffel, K., J.D. Davis, G. Rottman, J. Saltz, J. Dick, W. Merz and R. Miller. 1998. A graphical tool for ad hoc query generation. Proc. AMIA Symp. 503-507.

48. Charalambos, C., S. Swaboda, J.D.Dick, T. Perl, and P.A. Lipsett. 1998. Risk factors and clinical impact of central line infections in the surgical intensive care unit. Arch. Surg. 133: 1241-1246.

49. Parrish, N.M., F.P. Kuhajda, H.S. Heine, W.R. Bishai, and J.D. Dick. 1999 Antimycobacterial activity of cerulein and its effects on lipid biosynthesis. 1999. J. Antimicrob. Chemother. 43: 219-226.

50. Kirkpatrick, B.D., S.M. Harrington, D. Marcellus, C. Miller, D. Smith, J.D. Dick, L. Karanfil, T.M. Perl. 1999. An outbreak of vancomycin-dependent *Enterococcus faecium* in a bone marrow transplant unit. Clin. Infect. Dis. 29: 1268-73.

51. Singh, J., J. Dick, and M. Santosham. 2000. Colonization of the female urogenital tract with *Streptococcus pneumoniae* and implications for neonatal disease. Pediatric Inf. Dis. J. 19: 260-261.

52. Polack, F.P., D.C. Flayhart, M.L. Zahurak, J.D. Dick, and R.E. Willoughby. 2000 Colonization and antibiotic resistance of *Streptococcus pneumoniae* in children infected with the human immunodeficiency virus. Pediatric Infect. Dis. J. 19: 608-612.

53. Jones, P.B., N.M. Parrish, T.A. Houston, A.S. Stapon, N.P. Bansal, J.D. Dick, and C.A. Townsend. 2000. A new class of anti-tuberculosis agents. J. Med. Chem. 43: 3304-3314.

54. De Shazer, D., W.R. Byrne, R. Culpepper, G. Andrews, L. Hartman, G. Parker, H. Heine, A. Belani, J. Boyer, M. Barrera-Ora, C. Krause, A. Srinivasan, L. Karenfil, T. Perl, J. Bartlett, J. D. Dick, J. Bowles, J. Smith, A Danner, A. Hankinson, L. Edwards, and J. Roche. 2000. Laboratory-acquired human glanders-Maryland, May 2000. MMWR. 49: 532-535.

55. Truskinovshy, A.M., J.D.Dick, and G.M. Hutchins, 2001. Fatal infection after a bee sting. Clin. Inf. Dis. 32: E 36-38.

56. Hendrix, C.W., J.M. Hammond, S.M. Swoboda, W.G.Merz, S.M. Harrinton, T.M. Perl, J.D. Dick, D.M. Broschel, P.W. Halczendo, R.K. Pelz, L.E. Rocco, J.E. Conway, R.G. Brower, P.A. Lipsett. 2001. Surveillance strategies and impact of vancomycin-resistant enterococcal colonization and infection in critically ill patients. Ann. Surg. 233: 259-265.

57. Parrish, N.M., T. Houston, P.B. Jones, C. Townsend, and J.D. Dick. 2001. In vitro activity of a novel antimyco bacterial compound, N-octanesulfonylacetamide, and its effects on lipid and mycolic acid synthesis. Antimicrob. Agents Chemother. 45: 1143-1150.

58. Srinivasan, A., C.N. Kraus, D. Deshazer, P.M. Becker, J.D. Dick, L. Spacek, J.G.Barlett, W.R. Byrne and D.L. Thomas, 2001. Glanders in a military research microbiologist, N. Engl. J. Med. 345-(4)L 287-289.

59. Moss, W.J., C. Beers, E. Johnson, D.G. Nichols, T.M.Perl, J.D. Dick, M. A. Veltri, R.E. Willoughby Jr. 2002. Pilot study of antibiotic cycling in a pediatric intensive care unit. Crit. Care Med. 30:1877-1882.

60. Yang, S., S.Lin, G.D. Kelen, T.C. Quinn, J.D. Dick, C.A. Gaydos, and R.E. Rothman. 2002. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. J.Clin. Microbiol. 40: 3449-3454.

61. Moss, W.J., J.A. Sager, J.D. Dick and A. Ruff. 2003. *Streptomyces bikiniemsis* bacterimia. Emerg. Inf. Dis. 8. URL: http://www.cdc.gov/ncidod/EID/vol 9 no2/02-0275.htm

62. Sieradzki, K., T. Leski, J. Dick, L. Borio and A. Tomasz. 2003.Evolution of a vancomycin-intermediate *Staphylococcus aureus* strain "in vivo": Multiple changes in the antibiotic resistance phenotypes of a single lineage of methicillin – resistant *S. aureus* under the impact of antibiotics administered for chemotherapy. J. Clin. Microbiol. 41: 1687-1690.

63. Siberry, G.K., T. Tekle, K. Carroll, J. Dick. 2003. Failure of clindamycin treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) expressing inducible clindamycin resistance in vitro. Clin. Infect. Dis. 37:1257-1260.

64. Parrish, N.M., C.G. Ko, M.A. Hughes, C.A. Townsend, and J.D. Dick 2004. Effect of n-octanesulphonylacetamide (OSA) on ATP and protein expression in *Mycobacterium bovis* BCG. J. Anitmocrob chemother. 54(4): 722-729.

65. Parrish, N.M., C.G. Ko, J.D. Dick, P.B. Jones, and J.L.E. Ellingson. 2004. Growth, congored agar colony morphotypes and antibiotic susceptibility testing of *Mycobacterium avium* subspecies *paratuberculosis*. Clin. Med. Research 2: 107-114.

66. Hyon J.Y., M.J. Joo, S. Hose, D. Sinha, J.D. Dick, and T.P. O'Brien. 2004. Comparative efficacy of topical gatifloxacin with ciprofloxacin, amikacin, and clarithromycin in the treatment of experimental *Mycobacterium chelonae* keratitis. Arch. Ophthalmol. 122(8): 1166-1169.

67. Bettegowda, C., C.A. Foss, J. Cheong, Y. Wang, L. Diaz, N. Agrawal, J. Fox, J. Dick, L.H. Dang, S. Zhou, K.W. Kinzler, B. Vogelstein and M.G. Pomper. 2005. Imaging bacterial infections with radio labeled 1-(2'-fluoro-beta-D-arabinofuranosly)-5-iodouracil. Proc. Natl. Acad. Sci USA. 102 (4): 1145-1150.

68. Kim, D.H., W.J. Stark, T.P. O'Brien and J.D. Dick. 2005. Aqueous penetration and biological activity of moxifloxacin 0.5% ophthalmic solution and gatifloxacin 0.3% solution in cataract surgery patients. J. Ophthalmol. 112(11): 1992-1996.

69. Jain, S.K., A. Gupta, B. Glanz, J.Dick, and G.K. Siberry. 2005. Antimicrobial-resistant *Shigella sonnei*. Limited antimicrobial treatment options for children and challenges of interpreting in vitro azithromycin susceptibility. Ped. Infect. Dis. J. 24: 494-497.

70. Miller, N.S., J.D. Dick and W.G. Merz. 2006. Phenotypic switching in *Candida lusitaniae* on copper sulfate indicator agar: association with amphotericin B resistance and filamentation. J.Clin. Microbiol. 44(4): 1536.

71. Milstone, A.M., J.Dick, and G. Siberry. 2006. Treatment of Enterococcus faecalis ventriculoperitoneal shunt infection with linezolid. J. Neurosurgery. (submitted).

72. Parrish, N.M., C. G. Ko, and J.D. Dick. 2006. n-Decanesulfonylacetamide is bactericidal against anaerobically adapted *Mycobacterium bovis* BCG and has high sterilizing activity against rifampin-tolerant persisters in vitro. J. Antimicrob. Chemother. (submitted).

NON-PEER-REVIEWED ARTICLES

1. Karp, J.E. and J.D. Dick. 1993. Emergence of gram positive infections: Relationship to indwelling catheters and management during chemotherapy - induced aplasia. In: Shimpf, S.C. Klatersky, J, Eds. Recent Results: Cancer Research, Vol. 132. Springer-Verlag, Berlin-Hiedelberg, 221-229.

2. Dick, J.D. 1993. Gastrointestinal tract disorders: *Helicobacter pylori*. p. 156-158. In: McGraw-Hill Year Book of Science and Technology. 1993. McGraw-Hill, Inc. New York, NY.

3. Bartlett, J.G., T. Perl, J. Dick, W. Merz, P. Pham, S. Ray, J. Leslie, and P. Lipsett. 1998. Manual of Infectious Disease Care of Adults for Johns Hopkins Hospital.

EDITORIALS

1. Bartlett, J.G. and J.D. Dick. 2000. The controversy regarding routine anaerobic blood cultures. Am J. Med. 108: 505-506.

BOOK CHAPTERS, REVIEWS

1. Dick, J.D. 1990. *Helicobacter (Campylobacter) pylori*: A new twist to an old disease, p. 249-269. In L.N. Ornston, A. Balows, and E.P. Greenberg (ed.), Annual Review of Microbiology, Vol. 44. Annual Reviews, Inc., Palo Alto, California.

2. Popper, C., J. Dick, and P. Charache. 1991. Microbiology issues for critical care: Specimen procurement, technology assessment, and management implications. Critical Care Report. 2: 348-355.

3. Karp, J.E., W.G. Merz, and J.D. Dick, 1995. Empiric therapy and prevention of infection in the neutropenic patients. In Current Therapy in Internal Medicine, Mosby, New York.

4. Karp, J.E., W.G. Merz, and J.D. Dick. 1996. Emperic therapy and prevention of infection in the neutropenic patient. In Current Therapy in Internal Medicine, Mosby, New York.

5. Karp, J.E., W.G. Merz, and J.D. Dick. 1997. Empiric therapy and prevention of infection in the neutropenic patient. In, Current therapy in Adult Medicine, Mosby, New York.

6. Dick, J.D. and N.M. Parrish. 2000. Laboratory methods in the diagnosis of infections, p. 275-289. In K.E. Nelson, C. Masters and N. Graham (ed.) Infectious Diseases Epidemiology. Aspen Publishers, Inc., Gaithersburg, MD.

7. Srinivasan, A., J.D. Dick and T.M. Perl. 2002. Vancomycin resistance in *Staphylococcus* species. Clin. Microbiol. Rev. 15: 430-438.

8. Halon, A., M. Taylor, and J.Dick. 2006. Agar Dilution Susceptibility Testing. In Antimicrobial Susceptibility Testing Protocols. Steele-Moore et al. Taylor and Francis Group. Boca Raton, FL. (In press).

INVENTIONS, PATENTS, COPYRIGHTS:

1. U.S. Patent No. 5,614,551, James D. Dick, and Francis P. Kuhadja. 1997. Inhibitors of fatty acid synthesis as antimicrobial agents.

2. U.S. Patent Provisional Application Serial No. 60/056,272. C.A. Townsend, J.D. Dick, G.R. Pasternack, F.P. Kuhajda, and N. M. Parrish. Synthesis of Novel Compounds for the Treatment of Mycobacteria.

3. U.S. Patent Provisional Application, J.D. Dick, N.M. Parrish, and C.A. Townsend. Use of octanoic acid for the enhancement of growth in mycobacteria.

EXTRAMURAL SPONSORSHIP

GRANTS - active

1. Drug development for MDR - M.tb. 3-1-03 through 3-1-08. N.I.H. NIAID. 1UO1A154842-01. Total direct cost: $1,470,950. Current year direct cost $333, 565. P.I. - James D. Dick, Ph.D., 25% effort.

GRANTS – previous

1. Rational drug discovery for Mycobacterium avium complex and Mycobacterium tuberculosis. 81-98 through 8-1-03. N.I.H. R01 43846. Total direct cost: $1,019,435. Co-investigator, 10% effort. P.I. William R. Bishai, M.D.

2. Surveillance and control of nosocomial infections. 9-1-97 through 9-1-00. Centers for Disease Control. Technical salary and supply support-$44,600/year. Co-investigator; P.I.- Trish Perl, M.D.

CONTRACTS – previous

1. Ventria; Biosciences. Contract. 5-10-05 through 10-10-05. In vitro susceptibility testing of *Clostridium difficile* to lactoferrin and lysozyme. Total direct cost: % 20.000. Role: Co-P.I. 2% effort.

2. NIH. SBIR. R43. A10601434-01A2/ 7-1-05 through 12-31-05. Phase I. Rapid Susceptibility Testing of MDR *M. tuberculosis*. Total direct cost: %18,250. Role: Collaborator. 10% effort.

3. Direct fatty acid analysis by gas-liquid chromatography for identification of bacteria from blood culture, 5-1-01 through 4-30-03. NIAID SBIR consortium agreement. MIDI, Inc. Total direct cost: $58, 000. 10% effort.

4. Mechanism of action of N-octane sulfonyl acetamide in *Mycobacterium tuberculosis*. 3-3-01 through 12-31-02. FASgen, Inc, Total direct costs: $336,000. P.I James Dick, 11% effort.

5. Moxifloxacin regional and national *in vitro* susceptibility of common respiratory tract pathogens. 1999. Bayer Corp. $5,000.
6. Surveillance and control of nosocomial infections. 9-1-97 through 9-1-00. Centers for Disease Control. Technical salary and supply support-$44,600/year. Co-investigator; P.I. –Trish Perl, M.D.

7. Antibiotic resistance in ICUs. 1-1-01 through 7-31-01, Merck, Inc. $1,500. P.I. James Dick.

8. Antibiotic resistance in ICU'S. 1999, 1997, 1995, 1993, 1992, 1991, 1990. Merck, Inc. Total: $10,500.

9. Effect of *Bifidobacterium* adds to infant formula on the incidence of diarrheal disease. 1998. Gerber Foods. Technical salary and supply support - $15,000. Co-investigator. P.I. - P. Sevedra, M.D.

10. *In vitro* activity of SCH 27899, an everninomicin, against gram-positive bacteria. 1998. Schering Plough Research Institute. $5,000.

11. Effects of a probiotic to encourage the growth of *Bifidobacterium* on *Clostridium difficile* carriage and disease among the elderly. 1997, Ross foods. Technical salary and supply support. - $10,000. Co-investigators. P.I. - Richard Bennett, M.D.

12. Surveillance of *Bordetella pertussis* among health care workers. 1997 Maryland State Health Department. Technical Salary and supply support - $23,000. Co-investigator, P.I. - Trish Perl, M.D.

13. In vitro susceptibility testing of coagulase-negative staphylococci. 1998 National Committee on Clinical Laboratory Standards. CDC. $5,000.

14. In vitro susceptibility of cefpodoxime against clinical isolates of *Hemophilus influenzae* and *Streptococcus pneunomiae*. 1997. Upjohn Company. $5,000.

15. Multisite evaluation of Microscan MICSTREP frozen micro dilution panel for susceptibility testing of *Streptococcus pneumoniae* and other streptococci. 1996. Dade Microscan, Inc. $20,000.

16. Susceptibility of *Escherichia coli* and *Klebsiella pneumoniae* to cefoxitin and other selected antibiotics. Northeastern Regional Site. 1991. Merck, Inc, $30,000.

17. A multinuclear NMR approach to the determination of urea metabolism of *Campylobacter pylori* 1989. Interdisciplinary Committee on NMR. Johns Hopkins University. P.I. – James Dick, Ph. D. $6,910.

TEACHING

CLASSROOM INSTRUCTION

COURSE TITLE	DATES	ROLE
Infectious Diseases Epidemiology Department of Epidemiology, School of Public Health. 2 contact hours	2000 – present	Faculty
Nosocomial Infection. 4 credit Graduate Course, Department of Epidemiology. 1 contact hour	1997 – 2000	Faculty
Cellular and Molecular Medicine-Pathobiology. School of Medicine 4 contact hours	1997 - present	Faculty
Bacterial Pathogenesis II 4 credit Graduate course, Department of Molecular Microbiology & Immunology	1996 - present	Faculty
Principles of Bacterial Infection 3 credit Graduate course, Department of Molecular Microbiology and Immunology. 2 contact hours	1995- present	Faculty
Cellular and Molecular Mechanisms of Drug Action. Department of Pharmacology, School of Medicine. 2 contact hours	1995 – 1997	Faculty
Introduction to Pathology. Microbiology and Infectious Diseases Section. Required 2nd year medical student course. 42 contact hours	1993- present	Faculty
Microbiology & Microbial Genetics 4 credit Graduate Course-Department of Interdisciplinary Science Study	1992 - 1995	Director
Microbiology for Pathology Residents	1990 - present	Faculty

and Infectious Disease Fellows - 2 week course in July		
Bacterial Pathogenesis I - Credit Graduate course, Dept. of Molecular Microbiology & Immunology	1983 – 1995	Director

CLINICAL INSTRUCTION

Pediatric-monthly discussion with pediatric House officers concerning issues in Microbiology	1997 - present	Microbiology Faculty
Plate rounds - daily discussion of the Microbiology current infectious disease cases. Adult and pediatric infections disease rounding teams. 1/week	1990 - present	Microbiology Faculty

CME INSTRUCTION

Critical Issues in Laboratory Medicine	October 2-3, 1998	Microbiology Faculty
Critical Issues in Laboratory Issues in Medicine	May 28-29, 2003	Microbiology Faculty
Update on Ocular Infections Disease	May 21-22, 2004	Microbiology Faculty

MENTORING

ADVISEES - Postdoctoral

NAME	DATES	TITLE	ROLE	CURRENT POSITION
Nicole Parrish, Ph.D.	1999 - 01	Octanoic acid as a regulator of intermediate metabolism in mycobacteria	Mentor	Faculty, JHU
Nancy Miller, MD	1998 - 00	Elucidation of the biochemcial and molecular mechanisms of resistance to amphotericin B in *Candida lusitaniae*	Co-Mentor	Pathologist, Washington Hospital
V. Dixon King, MD	1992 - 93	Identification and antibiotic resistance in *Rhanella aquatilis* and vancomycin-resistant enterococcus	Co-Mentor	Pathologist St. Agnes Hospital
A. von Graevenitz, MD	1989	Sabatical - Cellular fatty acid analysis for identification of gram-positive rods	Mentor	Professor, Dept. of Medical Micro - Univ. of Zurich

ADVISEES – Predoctoral

NAME	DATES	TITLE	ROLE	CURRENT POSITION
Joan L. Valentine, MS	1991	Detection of *Helicobacter pylori* Using DNA hybridization techniques	Advisor	Education Coordinator Microbiology, JHH Medical School
Benjamin White, MS	1994	Endosymbiosis and its role in the Evolution of the eukaryotic cell	Advisor	
Holly L.H. Thomas, MS	1994	Evaluation of *Bacteriodes* isolation method as indicator of fecal contamination in selected pollution sources	Advisor	Research Technologist JHU
Nikki Parrish, Ph.D.	1999	Fatty acid synthesis inhibitors as antimycobacterial antibiotics	Advisor	Faculty, Pathology
Nancy T. Waites, MS	1996	Hepatitis C: an overview of the virus and possible treatments	Advisor	Research Technologist N.I.H.
Susan A. Smith, MHS.	1997	Drug resistance in *Streptococcus pneumoniae*	Advisor	Chief Microbiologist St. Andrews Hospital-Bermuda
Beverly A. Plunkett, MS	1997	Transmissible spongiform encephalopathies (Prion Disease)	Advisor	Research Technologist JHU
Peter Reese, MD	1997 - 99	Correlation of quantitative CRP with signs of sepsis in patients with blood cultures positive for coagulase-negative staphylococci	Advisor	Brigham & Women's Hospital
Jennifer Canfield MHS	2001	Effect of octanic acid on the growth of *Mycobacterium bovis* BCG	Advisor	Lead technologist JHH

THESIS COMMITTEES

NAME	DATES	DEGREE	
Jon J. Calomiris	1992-96	Ph.D.	Environmental Health Sciences - School of Medicine
Martin Sanders	1994-98	Ph.D.	Department of Immunology & Infectious Diseases School of Public Health
Nikki Parrish	1994-99	Ph.D.	Department of Immunology & Infectious Diseases School of Public Health

Girish Munavalli	1994-98	MHS	Department of Immunology & Infectious Diseases School of Public Health
Diana L. Guether	1994-96	Ph.D.	Department of Microbiology, Dental School, University of Maryland
Bruce H. Noden	1995-98	Ph.D.	Department of Molecular Microbiology & Immunology, School of Public Health
James E. Gomez	1995-99	Ph.D.	Department of Molecular Microbiology & Immunology, School of Public Health
William Nicholson	1995-99	Ph.D.	Department of Molecular Microbiology & Immunology, School of Public Health

EDITORIAL ACTIVITIES

Editorial Board:

Clinical and Diagnostic Laboratory Immunology	1995-2000
Letters in Applied Microbiology	1999-present

Ad hoc reviewer:
Journal of Clinical Microbiology
Antimicrobial Agents and Chemotherapy
Clinical Infectious Disease
Chest
Journal of Tropical Medicine
Journal of Antimicrobial Chemotherapy
Journal of Clinical Microbiology and
Infectious Disease
European Journal of Clinical Microbiology
and Infectious Disease

CLINICAL ACTIVITIES

CERTIFICATION:

1972 - Microbiologist, American Society for Clinical Pathology

1975 - General and Technical Supervisor Microbiology, Center for Disease Control

1998 - Diplomate, American Board of Medical Microbiology

SERVICE RESPONSIBILITIES

DIRECTOR OF BACTERIOLOGY 30% Effort

Technical Expertise
  Methods and Technology development
Administrative Responsibilities
  Personnel Development

ASSOCIATE DIRECTOR - MICROBIOLOGY DIVISION 10% Effort

Technical Expertise
QC
On-going Education
Methods and Technology development
Personnel Development
Lab Administration

ORGANIZATIONAL ACTIVITIES

Institutional Administrative Appointments

Antibiotic Subcommittee, Committee on Pharmacy and Therapeutics, Johns Hopkins Hospital	1983 - present
Committee on Admissions School of Medicine, Johns Hopkins University	1987 - 1990
Screening Committee, Committee on Admissions, School of Medicine, Johns Hopkins University	1994, 1996, 1999
Task Force on Bioterrorism Johns Hopkins Hospital	1999 - present
Antibiotic Committee, Oncology Center Johns Hopkins Hospital	1990 - Present

DEPARTMENTAL COMMITTEES (Recent)

Resident Selection and Training Committee
Diagnostic Immunology Search Committee
Pathology Grand Rounds Coordinator

PROFESSIONAL SOCIETIES:

American Society for Microbiology	1970 - present
Maryland Branch of the American Society for Microbiology	1970 - present
Executive Committee`	1988 - 1995
Medical Mycology Society of the Americas	1987 - present
Ocular Microbiology and Immunology Group, American Academy of Ophthalmology	1991 - present
American Association for the Advancement of Science	1991 - present
Fellow, Infectious Diseases Society of America	1992 - present

ADVISORY COMMITTEES, REVIEW GROUPS

NIH Ad hoc member – AIDS Discovery and Development of Therapeutics Study Section	2005
NIH Member – NIAID Units for HIV/AIDS clinical Trial Networks Special Emphasis Panel	2006
MRC – Reviewer – British Medical Research Council Grant - Review – Tuberculosis	2005-2006
Wellcome Trust – Reviewer – Intermediate Fellowship Award	2006
Inter – Institute Program for the Development of AIDs- Related Therapeutics; Review	
Committee, NCI/NIAID	2004
National Committee on Laboratory Standards Stenotrophomonas and Burkholderia Working Group Member	2003
Department of Veterans affairs Merit Review - Reviewer	2000, 2003

AWARDS, HONORS:

National Councilor,	1988-1990	Maryland Branch, American Society for Microbiology
Vice President	1991-1992	Maryland Branch, American Society for Microbiology
President	1993-1994	Maryland Branch, American Society for Microbiology
Fellow	1992-present	Fellow, Infectious Disease Society of America

INVITED TALKS

2006	NIAD Research Conference	n-Decanesulfonamide a new drug for the treatment of tuberculosis
2003	American Society of Clinical Laboratory Scientists. Philadelphia, PA.	Vancomycin resistance in staphylococci
2002	Biosecurity 2002, Harvard School of Public Health. Las Vegas, NE	Hospital laboratory approach for identification of bacterial agents of bioterrorism.

2001	Pathology Grand Rounds	*Mycobacterium tuberculosis* and the riddle of eights
2000	State of the Art in the Management of Urinary Tract Infections. NIH. Washington, DC	Diagnosis: Traditional and evolving tests
1999	Department of Pathology, Humboldt University, Berlin, Germany	Microbial diagnosis from biopsy tissue
1998	Pathology Grand Rounds	Drug development for *M. tuberculosis*
1997	Contemporary Issues in Pediatric Pharmacy, Mid-Atlantic Pediatric Pharmacotherapy Specialist, Baltimore, MD	Antimicrobial drug resistance
1995	Pathology Grand Rounds	Vancomycin resistance in Enterococci
1990	Current Issues in Infectious Diseases. Merck, Sharp and Dohme Albuquerque, NM	Antibiotic prophylaxes in the neutropenic patient
	Central Pennsylvania Microbiology Association. York, PA	Drug monitoring in the clinical microbiology laboratory
1988	Northeast Association of Clinical Microbiologists. Providence, RI	Diagnosis of *Helicobacter pylori*
1987	Prophylaxis of Gram negative infections in neutropenic patients. Merck. Zurich, Switzerland	Mechanisms of resistance of *Pseudomonas aeruginosa.*
	2nd Campylobacter workshop. NIH Keystone, CO	Identification of *Campylobacter pyloridis*
	Campylobacter pyloridis Symposium American Society for Microbiology. Atlanta, GA	In vitro culture and characteristics of *Campylobacter pyloridis*

Exhibit B

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2001, p. 1143–1150 Vol. 45, No. 4
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.4.1143–1150.2001

In Vitro Activity of a Novel Antimycobacterial Compound, *N*-Octanesulfonylacetamide, and Its Effects on Lipid and Mycolic Acid Synthesis

NIKKI M. PARRISH,[1] TODD HOUSTON,[2†] PAUL B. JONES,[2‡] CRAIG TOWNSEND,[2] AND JAMES D. DICK[1,3*]

Department of Pathology, School of Medicine,[1] Department of Molecular Microbiology and Immunology, School of Public Health,[3] and Department of Chemistry, Johns Hopkins School of Arts and Sciences,[2] Johns Hopkins University, Baltimore, Maryland

Received 29 September 2000/Returned for modification 8 November 2000/Accepted 22 January 2001

β-Sulfonyl carboxamides have been proposed to serve as transition-state analogues of the β-ketoacyl synthase reaction involved in fatty acid elongation. We tested the efficacy of *N*-octanesulfonylacetamide (OSA) as an inhibitor of fatty acid and mycolic acid biosynthesis in mycobacteria. Using the BACTEC radiometric growth system, we observed that OSA inhibits the growth of several species of slow-growing mycobacteria, including *Mycobacterium tuberculosis* (H37Rv and clinical isolates), the *Mycobacterium avium* complex (MAC), *Mycobacterium bovis* BCG, *Mycobacterium kansasii*, and others. Nearly all species and strains tested, including isoniazid and multidrug resistant isolates of *M. tuberculosis*, were susceptible to OSA, with MICs ranging from 6.25 to 12.5 μg/ml. Only three clinical isolates of *M. tuberculosis* (CSU93, OT2724, and 401296), MAC, and *Mycobacterium paratuberculosis* required an OSA MIC higher than 25.0 μg/ml. Rapid-growing mycobacterial species, such as *Mycobacterium smegmatis*, *Mycobacterium fortuitum*, and others, were not susceptible at concentrations of up to 100 μg/ml. A 2-dimensional thin-layer chromatography system showed that OSA treatment resulted in a significant decrease in all species of mycolic acids present in BCG. In contrast, mycolic acids in *M. smegmatis* were relatively unaffected following exposure to OSA. Other lipids, including polar and nonpolar extractable classes, were unchanged following exposure to OSA in both BCG and *M. smegmatis*. Transmission electron microscopy of OSA-treated BCG cells revealed a disruption in cell wall synthesis and incomplete septum formation. Our results indicate that OSA inhibits the growth of several species of mycobacteria, including both isoniazid-resistant and multidrug resistant strains of *M. tuberculosis*. This inhibition may be the result of OSA-mediated effects on mycolic acid synthesis in slow-growing mycobacteria or inhibition via an undescribed mechanism. Our results indicate that OSA may serve as a promising lead compound for future antituberculous drug development.

Tuberculosis continues to be the leading cause of death worldwide due to an infectious agent (8). Approximately 8 million new active cases arise each year, with about 3 million deaths (8). Of equivalent concern has been the emergence of multidrug-resistant *Mycobacterium tuberculosis*. As a result, newly infected individuals no longer have the assurance that prophylaxis with isoniazid (INH) will eliminate infection or that active disease will be treatable with our current arsenal of drugs. In addition, therapies for the treatment of atypical mycobacterial infections in immunocompromised patients are limited (24). Thus, the development of new drugs is essential in combating both drug resistant *M. tuberculosis* and opportunistic infections with atypical mycobacteria, such as the *Mycobacterium avium* complex (MAC).

Potential new targets for antimycobacterial drug development may exist among the synthetic enzymes needed to make the unique lipids produced by mycobacteria, such as mycolic acids. These high-molecular-weight, α-alkyl, β-hydroxy fatty acids comprise the single largest component of the mycobacterial cell envelope (3, 4, 9, 10, 29, 30, 37). They are found in free lipids as trehalose mono- and dimycolate and esterified to the arabinogalactan matrix of the mycobacterial cell wall (5, 10). They are vital for the growth and survival of mycobacteria, as evidenced by the bactericidal properties of mycolic acid inhibitory drugs, such as isoniazid and ethionamide (1, 2, 32, 33, 43, 44, 47, 48, 51, 53–58).

Synthesis of mycolic acids and other mycobacterial lipids requires a variety of fatty acid synthase and elongation enzymes (7, 10, 23). Although the synthesis of fatty acids is essentially the same at the primary chemical level, fatty acid synthases (FAS) are organized into two types. In Type I FAS (FAS I), most often found in eukaryotes, the individual enzymatic reactions are contained in one multienzyme complex. In Type II FAS (FAS II), commonly found in prokaryotes, the enzyme functions are carried out by seven individual proteins. Mycobacteria are known to possess both FAS I and II (6, 7, 23). Thus, inhibition of these enzymes, especially those involved in chain elongation of unique mycobacterial fatty acids, may provide novel targets for drug design.

In the past, characterization of FAS has been aided through the use of two natural product inhibitors of FAS components,

* Corresponding author. Mailing address: Johns Hopkins Medical Institutions, Department of Pathology/Division of Medical Microbiology, 600 N. Wolfe St., Baltimore, MD 21287. Phone: (410) 955-5077. Fax: (410) 614-8087. E-mail: jdick@jhmi.edu.

† Present address: Department of Chemistry, Virginia Commonwealth University, Richmond, Va.

‡ Present address: Department of Chemistry, Wake Forest University, Winston-Salem, N.C.

FIG. 1. Structure of OSA.

cerulenin and thiolactomycin (15, 16, 36, 39–42, 46). Cerulenin is a potent inhibitor of both FAS I and FAS II systems while thiolactomycin inhibits only synthases of the FAS II variety. Activity of both of these inhibitors on the mycolic acids of mycobacteria has recently been described (25, 42, 50). Although cerulenin and thiolactomycin are structurally different, both compounds inhibit the two-carbon homologation catalyzed by the β-ketoacyl synthase, the condensing enzyme required for fatty acid biosynthesis. Specifically, cerulenin irreversibly inhibits the β-ketoacyl synthase (20, 39, 40), while thiolactomycin inhibits both the β-ketoacyl–acyl carrier protein (ACP) synthase and acetyl coenzyme A:ACP transacylase (15). β-Sulfonyl carboxamides were designed to mimic the transition state of the reaction catalyzed by the β-ketoacyl synthase. In the following study we evaluated the in vitro activity of one of these compounds, *N*-octanesulfonylacetamide (OSA), on a variety of mycobacteria and specifically evaluated its effects on lipid and mycolic acid synthesis in *Mycobacterium bovis* BCG and *Mycobacterium smegmatis*.

MATERIALS AND METHODS

Synthesis of OSA. The synthesis of alkyl sulfoxides and sulfones has been described previously (22). Briefly, OSA was synthesized in three steps from commercially available materials. Octyl bromide and methyl thioglycolate were reacted together to yield methyl 3-thioundecanoate. This sulfide was then oxidized to the sulfoxide by using 3-chloroperoxybenzoic acid. OSA was obtained from the ammonylysis of the methyl ester. Overall yield was 70% following crystallization of the final product (Fig. 1).

Mycobacteria. *M. tuberculosis* strains H37Rv and CSU93 (52), *M. bovis* (ATCC 35734), *M. bovis* BCG (Pasteur strain, ATCC 35734), *Mycobacterium kansasii* (ATCC 12478), *Mycobacterium paratuberculosis* (ATCC 19698), and *M. smegmatis* (mc^2 6 1-2c) (53) were utilized as reference strains. Clinical and other isolates were speciated using standard methods (38) and included MAC, *Mycobacterium fortuitum*, *Mycobacterium chelonei*, *Mycobacterium abscessus*, and both INH- and multidrug-resistant clinical isolates of *M. tuberculosis*.

Susceptibility testing. Susceptibility testing and determination of MICs for *M. tuberculosis*, *M. bovis*, *M. kansasii*, and *M. bovis* BCG were done using the BACTEC radiometric growth system (Becton Dickinson, Sparks, Md.) and a standardized method (42, 49). Initial stock solutions (1 mg/ml) and subsequent dilutions of OSA, cerulenin (Sigma, St. Louis, Mo.), and thiolactomycin (generously provided by T. Yoshida) were prepared in dimethyl sulfoxide (Sigma). A modification of this procedure adopted by The National Jewish Center for Immunology and Respiratory Medicine was used to determine MICs for MAC (17). Susceptibility testing of *M. paratuberculosis* was accomplished by varying the standard BACTEC protocol to include the addition of mycobactin J (Allied Monitor, Fayette, Mo.) to commercially prepared 12B media (Becton Dickinson). Initial mycobactin J solutions (2 mg/ml) were brought up in 95% ethanol and diluted in sterile distilled water to a concentration of 40 μg/ml. Mycobactin J was then added to each BACTEC vial (final concentration = 1.0 μg/ml) along with OSA. All primary drugs were purchased from Becton Dickinson. Susceptibilities and MIC determinations of specific inhibitors for *M. smegmatis*, *M. fortuitum*, *M. chelonei*, and *M. abscessus* were established by broth dilution using Middlebrook 7H9-ADC incubated at 37°C for 4 days.

Treatment of cultures with OSA and lipid pulse labeling. BCG and MAC cells were grown in M7H9-ADC-Tween (Difco, Detroit, Mich.) to early log phase. From this, a 1.0 McFarland suspension was prepared and diluted to yield a final concentration of 3×10^7 cells/ml in a total volume of 50 ml in M7H9-ADC-Tween. Cultures were aerated and incubated at 37°C for 24 h (approximately 1 generation time). Each inhibitor was added at its MIC (final concentrations: thiolactomycin, 25.0 μg/ml [BCG] and 75.0 μg/ml [*M. smegmatis*]; OSA, 6.25 μg/ml [BCG], 25.0 μg/ml [MAC], and 100 μg/ml [*M. smegmatis*]), and cultures were incubated under the same conditions for approximately 1 generation time (BCG and MAC, approximately 24 h; *M. smegmatis*, approximately 5 h). Subsequently, 1 μCi of [1,2-^{14}C]acetic acid (Amersham, Arlington Heights, Ill.)/ml was added and the cultures were incubated as before for an additional 24 h. In order to demonstrate a concentration-dependent effect of OSA on mycolic acid synthesis in BCG, lipid pulse labeling was also performed at OSA concentrations of 12.5 and 25.0 μg/ml. A slight variation of this protocol was used for labeling in *M. smegmatis* cells. Since this species of mycobacteria was not susceptible to OSA, the highest concentration tested (100 μg/ml) in this study was used for labeling purposes. Additionally, a 0.5 McFarland suspension was done with an initial incubation time of 10 h prior to the addition of compound and subsequent incubations of 5 h each (based on a doubling time of 3 to 5 h) following the addition of drug and label, respectively. All assays were performed in duplicate.

Preparation of extractable mycobacterial lipids. Extractions were performed as previously described (13, 34, 42). Briefly, 100 to 150 mg (wet weight) of cells was suspended in methanolic saline (methanol–0.3% aqueous NaCl [100:10, vol/vol] [2 ml]) and extracted three times with petroleum ether, yielding nonpolar extractable lipids. The remaining cells and residual aqueous phase were boiled for 5 min, cooled for 5 min at 37°C, and extracted with monophasic chloroform–methanol–0.3% NaCl (90:100:30, vol/vol; used once) and chloroform–methanol–0.3% NaCl (50:100:40, vol/vol; used twice). All extracts were subsequently dried under N_2 at room temperature. The defatted cells containing saponifiable lipids were saved.

Mycolic acid extraction and preparation of MAMES and FAMES. Extractions of mycobacterial mycolic acids were performed as previously described (13, 35, 42). Briefly, 50-ml cultures of *M. smegmatis*, BCG, or MAC cells were harvested by centrifugation at 3,000 × *g* for 10 min. Equal volumes of cells (100 to 150 mg [wet weight]) were extracted to remove polar and nonpolar extractable lipids (13, 42). The resulting defatted cells containing bound mycolic acids and other saponifiable lipids were subjected to alkaline hydrolysis in methanol (1 ml), 30% KOH (1 ml), and toluene (0.1 ml) at 75°C overnight and subsequently cooled to room temperature (13, 42). The mixture was then acidified to pH 1 with 3.6% HCl and extracted three times with diethyl ether. Combined extracts were dried under N_2. Mycolic acid methyl esters (MAMES) and other long-chain fatty acids (fatty acid methyl esters [FAMES]) were prepared by mixing dichloromethane (1 ml), a catalyst solution (1 ml) (14), and iodomethane (25 ml) for 30 min; centrifuging; and discarding the upper phase. The lower phase was dried under N_2.

[1,2-^{14}C]acetate incorporation into mycobacterial lipids. Incorporation of [1,2-^{14}C]acetate into polar and nonpolar extractable and saponifiable lipid fractions was determined by scintillation counting and expressed in counts per minute (cpm) (Beckman LS6500 multi-purpose scintillation counter).

Analysis of MAMES and FAMES. Mycobacterial saponifiable extracts containing MAMES and FAMES were dissolved in chloroform and equal counts (in counts per minute) of each sample were loaded onto thin-layer chromatography (TLC) plates (20- by 20-cm silica gel G, 250-μm-diamter analytical plates; Analtech, Newark, Del.). Samples were subsequently subjected to a 2-dimensional solvent system (petroleum ether [bp 60 to 80°C]–acetone [95:5, vol/vol]) in the first dimension [three times] and toluene-acetone [97:3, vol/vol] in the second dimension [one time]).

Data analysis. Mycolic acids of each species of mycobacteria were identified according to methods described by Dobson et al. (13, 42). Visualization and comparison of thin-layer chromatograms were done using a Fuji Systems (Fujix BAS 1000) phosphorimager. Spots were quantified using NIH Image (version 1.57; National Institutes of Health, Bethesda, Md.) software programs. Due to the nonequivalency of the counts per minute as determined by scintillation counting and phosphor counts as determined by phosphorimaging, relative intensities of chromatographed compounds were calculated for each TLC plate on the basis of total number of phosphor counts per plate. Phosphor counts for MAMES and FAMES were normalized for each TLC plate pair (control and inhibitor treated). The difference in normalized phosphor counts between each control and inhibitor treated pair represents the percent change.

Electron microscopy. All chemicals and reagents for electron microscopy were obtained from Electron Microscopy Sciences, Ft. Washington, Pa. Cultures (50 ml) of BCG were grown to early log phase (optical density at 600 nm, ~0.2), at which time OSA (100 μg/ml) or diluent (dimethyl sulfoxide) was added to treated and control cultures, followed by additional aeration and incubation at 37°C for 24 h. Cells were harvested by low-speed centrifugation and washed in 0.1 M cacodylate (CACO) buffer (pH 7.3). Washed cells were then fixed in 0.1 M CACO buffer (pH 7.3) containing 2.0% glutaraldehyde–osmium tetroxide (1:1, vol/vol) for 45 min at 4°C (11, 28, 45). Secondary fixation was done at 4°C

TABLE 1. Susceptibility of different species of mycobacteria to OSA

Organism	MIC (μg/ml) of OSA
M. bovis	6.25
M. bovis BCG	6.25
M. kansasii	12.5
M. avium complex	25.0
M. paratuberculosis	25.0
M. smegmatis	>100
M. fortuitum	>100
M. chelonei	>100
M. abscessus	>100

overnight in 4.0% formaldehyde–1.0% glutaraldehyde. Samples were post-fixed at room temperature for 1 h in 0.1 M CACO buffer containing 1% tannic acid and dehydrated through a graded ethanol series of 50, 70, 95 (twice), and 100% (three times). Subsequently, samples were infiltrated at room temperature with a series of Spurrs resins (Spurrs-ethanol [1:1, vol/vol; 2 h], Spurrs-ethanol [2:1, vol/vol; 2 h], and pure Spurrs [overnight]), and blocks were polymerized at 60°C for 48 h. Sections were cut on a Sorvall MT2B microtome, and 80-nm-thick sections were picked up on 200-mesh copper grids and stained with uranyl acetate and lead citrate. Prepared samples were then analyzed on a Hitachi HU12A electron microscope.

RESULTS

In vitro susceptibilities. Tables 1 and 2 show the susceptibility of various mycobacterial species and strains to OSA using the BACTEC radiometric growth system. Nearly all strains of *M. tuberculosis* and other slow-growing mycobacterial species, such as *M. bovis, M. bovis* BCG, and *M. kansasii*, were susceptible to OSA, with MICs ranging from 6.25 to 12.5 μg/ml. Only three clinical isolates of *M. tuberculosis* (CSU 93, 42-1C9383, and 10129-3), the MAC, and *M. paratuberculosis* required a higher OSA MIC of 25 μg/ml. None of the rapid-growing mycobacterial species tested, *M. smegmatis, M. fortuitum, M. chelonei*, and *M. abscessus*, were susceptible to OSA at concentrations up to 100 μg/ml. Resistance to known antimycobacterial agents did not give cross-resistance to OSA. Three clinical isolates of *M. tuberculosis*, resistant to either INH alone or INH plus rifampin, ethambutol, streptomycin, and pyrazinamide (19), were found to be susceptible to OSA, with MICs of 12.5 and 6.25 μg/ml, respectively. Similar results were obtained for cerulenin as previously reported (42). BCG and *M. smegmatis* were susceptible to thiolactomycin at a MIC of 25 and 75 μg/ml, respectively.

Overall effects of OSA on mycobacterial lipids. Labeling assays were conducted at the calculated MIC (6.25 μg/ml) of OSA for BCG and at the highest concentration of compound tested for *M. smegmatis*. This particular concentration of OSA is not equivalent to the lethal concentration of compound in mycobacteria, as evidenced by continued $^{14}CO_2$ production in the radiometric susceptibility test system, which indicated continued metabolism, albeit at a reduced level relative to those of controls. OSA had no significant effect on [^{14}C]acetate incorporation into nonpolar extractable or polar extractable lipids at a concentration of 6.25 μg/ml, the calculated MIC for *M. bovis* BCG, or 100 μg/ml for *M. smegmatis* (Fig. 2). A moderate decrease in the incorporation of label was observed in saponifiable lipids in OSA-treated BCG. However, this change was not statistically significant. Label incorporation into the same lipid fraction in *M. smegmatis* was unaltered by exposure to OSA (Fig. 2). In order to demonstrate a concentration-dependent effect of OSA on lipid metabolism, studies were run at 12.5 μg/ml (two times the MIC) and 25.0 μg/ml (four times the MIC). At these higher concentrations, there was a dose-dependent decrease of label incorporation in the saponifiable lipid fraction with a concomitant increase of label in the nonpolar extractable fraction (data not shown).

Effect of OSA on mycolic acid synthesis as compared with thiolactomycin. Qualitative and quantitative analysis of mycobacterial saponifiable lipids containing MAMES was performed using [^{14}C]acetate pulse-labeling with 2-dimensional TLC and phosphorimaging. Differences in the effects of OSA and thiolactomycin were found between BCG and *M. smegmatis*. OSA treatment in BCG resulted in inhibition of all mycolic acids commonly found in this mycobacterial species (Fig. 3; Table 3). This decrease or percent change in both α- and keto-mycolates reached greater than 90% with an OSA concentration of four times the MIC. Similar results were demon-

TABLE 2. Activities of OSA and first-line antimycobacterial drugs against various strains of *M. tuberculosis*[a]

M. tuberculosis strain	INH		RIF	EMB	STR	PZA	OSA		
	0.1 μg/ml	0.4 μg/ml	(2.0 μg/ml)	(2.0 μg/ml)	(2.0 μg/ml)	(100 μg/ml)	6.25 μg/ml	12.5 μg/ml	25.0 μg/ml
H37Rv	S	S	S	S	S	S	S	S	S
5D5178	S	S	S	S	S	S	S	S	S
1D4924	S	S	S	S	S	S	S	S	S
1H1337	S	S	S	S	S	S	S	S	S
42401315	S	S	S	S	S	S	S	S	S
6T2709	S	S	S	S	S	S	S	S	S
1T2768	S	S	S	S	S	S	S	S	S
TBL54 EP066	R	R	R	R	R	R	S	S	S
7D5245	S	S	S	S	S	S	S	S	S
OT2769	S	S	S	S	S	S	R	S	S
42-1C9383	R	R	S	S	S	S	R	S	S
10129-3	R	R	S	S	S	S	R	S	S
CSU93	S	S	S	S	S	S	R	R	S
OT2724	S	S	S	S	S	S	R	R	S
401296	S	S	S	S	S	S	R	R	S

[a] S, susceptible; R, resistant; RIF, rifampin; EMB, ethambutol; STR, streptomycin; PZA, pyrazinamide.

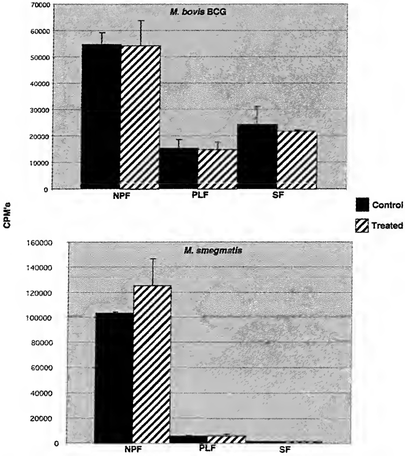

FIG. 2. Effects of OSA on the synthesis of various lipid fractions in *M. bovis* BCG and *M. smegmatis*. Incorporation of [1,2-^{14}C]acetate into extractable polar, nonpolar, and saponifiable lipids in the presence and absence of OSA. Abbreviations: NPF, nonpolar extractable lipids; PLF, polar extractable lipids; SF, saponifiable lipids, including mycolic acids. Concentrations of OSA used: for *M. smegmatis*, 100 μg/ml; for *M. bovis* BCG, 12.5 μg/ml. Differences were not statistically significant.

strated for MAC (data not shown). However, in *M. smegmatis*, individual mycolate classes were only slightly inhibited following exposure to OSA (Fig. 4; Table 3). Other lipids present in the saponifiable fraction included FAMES. No appreciable change was observed in this lipid class following OSA treatment in either of the two mycobacterial species characterized in this study (Fig. 3 and 4; Table 3).

Thiolactomycin caused a decrease in all mycolate species in BCG (Fig. 3; Table 3) and *M. smegmatis*. However, while thiolactomycin uniformly inhibited all mycolate species in BCG, in *M. smegmatis*, a differential effect was observed between individual mycolic acid classes. Both α-mycolates and epoxymycolates were nearly completely diminished (95 and 87%, respectively), whereas α′-mycolates were less affected (57%) (Fig. 4; Table 3). In contrast to OSA, FAMES accumulated in both BCG and *M. smegmatis* following treatment with thiolactomycin.

Transmission electron microscopy of OSA-treated BCG. Inhibition of mycolic acid synthesis is known to disrupt the mycobacterial cell wall. Figure 5 shows transmission electron micrographs of OSA-treated BCG cells during cell division. Control organisms exhibited an intact cell wall and clearly defined septum, whereas in the presence of OSA, cell wall synthesis was disrupted with incomplete septum formation. In addition, outer-wall-associated lipids appeared to be dispersed from the electron transparent zone of the mycolic acids.

DISCUSSION

In this study, we demonstrate that OSA, a compound designed to inhibit fatty acid synthesis by mimicking the transition state of the β-ketoacyl synthase, is inhibitory for a broad range of slow-growing mycobacterial species, including multidrug-resistant *M. tuberculosis*. OSA treatment reduced mycolic acid accumulation in BCG and MAC cells, presumably by its effect on FAS systems in these mycobacteria. Cross-resistance was not observed for isolates resistant to isoniazid, rifampin, ethambutol, streptomycin, and pyrazinamide. A comparison of isoniazid, a known inhibitor of mycolic acid synthesis, and OSA revealed pertinent information. Isoniazid has been shown to inhibit both InhA, an enoyl-ACP reductase involved in fatty acid elongation, and KasA, a β-ketoacyl–ACP synthase (2, 12,

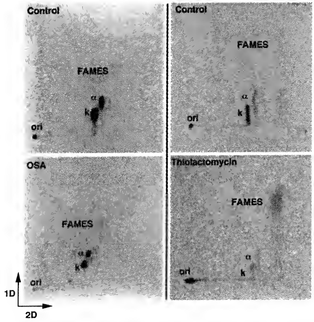

FIG. 3. Two-dimensional TLC showing the comparative effects of OSA and thiolactomycin on the mycolic acids of *M. bovis* BCG. First dimension, petroleum ether (bp 60 to 80°C)–acetone (95:5, vol/vol; three times); second dimension, toluene-acetone (97:3, vol/vol; one time). Abbreviations: ori, origin; α, α-mycolate; k, keto-mycolate. Equivalent counts per minute of the saponifiable lipid fraction were spotted at each origin.

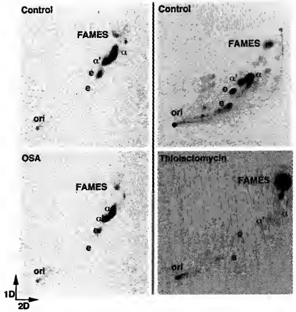

FIG. 4. Two-dimensional TLC showing the comparative effects of OSA and thiolactomycin on the mycolic acids of *M. smegmatis*. First dimension, petroleum ether (bp 60 to 80°C)-acetone (95:5, vol/vol; three times); second dimension, toluene-acetone (97:3, vol/vol; one time). Abbreviations: ori, origin; α, α-mycolate; α′, α′-mycolate; e, epoxymycolate. Equivalent counts per minute of the saponifiable lipid fraction were spotted at each origin.

28, 31, 32). INH resistance has been associated with mutations in both of these genes, as well as the KatG gene, which encodes the mycobacterial catalase-peroxidase (54, 55, 57, 58). In this study, the mechanism of INH resistance for the *M. tuberculosis* isolates used was not determined. Of relevance is the finding that OSA inhibited the growth of INH-resistant *M. tuberculosis* (>0.04 μg/ml) as well as MAC, typically resistant to INH (>2.5 μg/ml) (19). This observation suggests that the inhibition of mycolic acid synthesis by OSA may be due to interaction with an enzymatic target different from that of INH, indicating a novel and possibly unexploited mechanism of action of OSA in *M. tuberculosis*.

Although OSA was designed to inhibit β-ketoacyl synthases, it has not been tested as an inhibitor of isolated FAS. In an earlier, related study conducted in our laboratory, structurally related sulfones and sulfoxides were found to inhibit FAS I isolated from *M. smegmatis* (41). Several of these compounds were both FAS I inhibitors and active against *M. tuberculosis* H37Rv in the radiometric growth assay. However, the correlation was not exact and issues of cell wall permeability and solubility prevented direct comparison of the data. OSA was selected for further study based on its solubility and its performance in whole-cell assays.

The differential susceptibility to OSA observed between slow- and rapid-growing mycobacterial species argues for the presence of unique targets in BCG and MAC. *M. smegmatis* may contain the same OSA target as BCG and MAC but possesses alternate cell wall compounds that permit survival in spite of OSA inhibition. Another potential possibility is the requirement for alteration, i.e., activation, of OSA prior to its interaction with the target protein(s) which may occur in slow-growing mycobacteria but not in rapid-growing species. However, the most probable interpretation is that differences in the mycolic acid biosynthetic pathway may exist between mycobacterial species. This possibility is further strengthened when the work of other investigators is considered in conjunction with our own. For example, InhA, a long chain, enoyl-ACP-dependent reductase involved in fatty acid elongation, is present in both *M. smegmatis* and *M. tuberculosis* (31). Several studies have revealed compelling evidence that InhA is the target for KatG-activated INH in *M. smegmatis* (12, 44). However, additional investigations suggest that this particular enzyme is not the principal target for KatG-activated INH in *M. tuberculosis*

TABLE 3. Effects of OSA and thiolactomycin on the mycolic acids of *M. bovis* BCG and *M. smegmatis*

Organism	Mycolic acid species	Inhibition (% change)	
		OSA	TLM
M. bovis BCG	α	−56	−69
	keto	−52	−79
	FAMES	—[a]	+147
M. smegmatis	α	−19	−95
	α′	−18	−57
	epoxy	−8	−87
	FAMES	—	+134

[a] —, no appreciable change was observed.

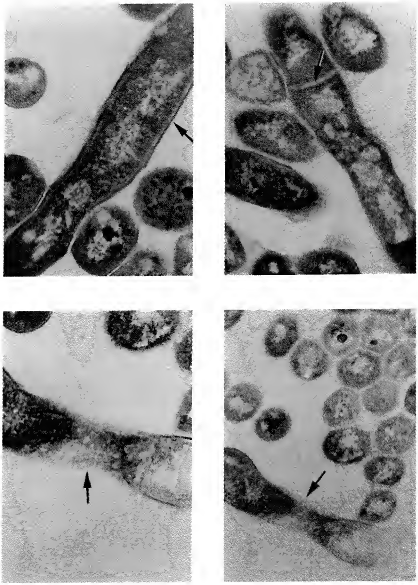

FIG. 5. Transmission electron microscopy of OSA-treated *M. bovis* BCG (6.25 μg/ml). (Top) Intact cell wall ultrastructure (left) (magnification, ×117,000) and completed septum (right) (magnification, ×108,000). (Bottom) Disrupted cell wall ultrastructure (left) (magnification, ×130,000) and incomplete septum formation (right) (magnification, ×78,000).

(31, 32). This discrepancy may reflect inherent differences in mycolate biosynthesis between the two organisms (31, 32, 42). Additionally, previous studies in our laboratory examining the effect of cerulenin on mycolate synthesis in *M. smegmatis* and BCG revealed clear differences in mycolic acid profiles between these two mycobacterial species following inhibitor exposure. Not only did the changes in mycolate synthesis differ between BCG and *M. smegmatis*, they were in direct opposition. For instance, completed mycolates decreased in BCG following exposure to cerulenin, whereas in *M. smegmatis*, all mycolate species increased, again suggesting that inherent differences in the mycolate biosynthetic pathway between the two species are responsible for these disparate responses (42).

This possibility is further strengthened by the differential effect of thiolactomycin on individual mycolic acid classes in *M. smegmatis*. In both the present study and that of other investigators (50), exposure to thiolactomycin resulted in substantially decreased amounts of α-mycolates and epoxymycolates, with a minor decrease in α′-mycolates (49). The authors of the previous study suggested that potential targets for thiolactomycin in this mycobacterial species include an elongation enzyme leading from either the $C_{24:1}\Delta5$ intermediate or from the shorter-chain α′-mycolates to the longer α-mycolates and oxygenated mycolates (50). In view of the current experimental evidence, the latter seems to be the more likely possibility. It should be noted that α′-mycolates, commonly found in rapid-growing mycobacteria, are not present in BCG and other slow-growing mycobacteria characterized in this study (18). Thus,

while thiolactomycin treatment of BCG and *M. smegmatis* resulted in inhibition of both α-mycolates and oxygenated mycolates, the presence of α′-mycolic acids in the latter case may partially explain the differences observed in MICs between the two mycobacterial species (for BCG, 25.0 μg/ml; for *M. smegmatis*, 75 μg/ml) and indicate that disparities in mycolate biosynthesis may exist between BCG and *M. smegmatis*. One could speculate that such differences may extend to other slow- versus rapid-growing mycobacterial species.

Differences in mycolic acid profiles following OSA treatment in BCG and *M. smegmatis* were also noted. In the present study, all mycolic acids were significantly and uniformly inhibited in OSA-treated BCG (α- and keto-mycolates). This inhibition increased with an increasing concentration of OSA. In contrast, the effect of OSA on the mycolates of *M. smegmatis* (α′, α, and epoxy) was negligible and not inhibitory to growth. Previous studies have suggested the existence of multiple ACP-dependent FAS II systems in mycobacteria, responsible for not only fatty acid biosynthesis but also mycolic acid biosynthesis (2, 32, 42, 44). Such systems could be envisioned to interact with separate and distinct β-ketoacyl–ACP synthases as well as other enzymes involved in biosynthetic reactions of this type, including β-ketoacyl–ACP reductases, β-hydroxyacyl–ACP dehydratases, and β-enoyl–ACP reductases. A biological precedent for the existence of such enzymes has been described for *Esherichia coli*, in which multiple β-ketoacyl–ACP synthases have been found (21, 26, 27). Although thiolactomycin was originally thought to inhibit all three β-ketoacyl–ACP synthases in *E. coli*, recent evidence suggests that the principal target of thiolactomycin in this particular organism may be only β-ketoacyl–ACP I (25). Since OSA was designed to inhibit the β-ketoacyl synthase by mimicking the transition state of the reaction catalyzed by this enzyme, this compound could in theory inhibit both the multifunctional FAS I and monofunctional FAS II mycobacterial systems, as in the case of cerulenin. However, in this study, additional assays were performed which were designed to indirectly determine FAS I activity in the presence of each inhibitor by measuring phospholipid production. Only cerulenin, known to inhibit both FAS I and FAS II systems, interfered with phospholipid synthesis in either BCG or *M. smegmatis* (42). Neither thiolactomycin, active only against FAS II systems, nor OSA inhibited phospholipid synthesis in either of the two mycobacterial species tested (data not shown), suggesting that the principal target of OSA may lie in an ACP-dependent FAS II system. In addition, previous work in our laboratory and others has demonstrated that cerulenin and thiolactomycin strongly inhibited [^{14}C]acetate incorporation into other extractable mycobacterial lipids, a finding consistent with the known mechanism of action of both inhibitors. In contrast, OSA inhibited only mycolic acids, with no appreciable change in label incorporation in any of the other mycobacterial lipid classes tested. This distinction suggests the presence of a unique and highly specific target for this compound in slow-growing mycobacteria. Such a target may involve an as-yet-unidentified enzyme or enzyme system present in slow-growing mycobacteria which is not present or inactive in rapid-growing species.

Additional information was obtained by careful analysis of FAMES in OSA-treated BCG and *M. smegmatis*. Other investigators have determined that these lipids most likely represent saturated alkyl intermediate(s) in mycolic acid synthesis (31). While 2-dimensional TLC of OSA-treated BCG revealed that mycolic acid synthesis was inhibited, no apparent effect was seen in the FAMES present in this fraction when the compound was used at the MIC (6.25 μg/ml). A similar observation was noted in OSA-treated *M. smegmatis*. However, at four times the MIC, OSA treatment of BCG resulted in an increase in label incorporation into extractable nonpolar lipids. This may suggest that a noncovalently bound, extractable intermediate in mycolate synthesis accumulates following OSA treatment, an effect intensified with higher concentrations (four times the MIC) of compound. In contrast, FAMES increased in thiolactomycin-treated BCG, while mycolic acids decreased, a finding consistent with that of earlier studies using cerulenin (42). Thus, in BCG, while completed mycolates decreased with OSA, thiolactomycin, and cerulenin (42), the changes in FAMES were clearly not the same, suggesting that inhibition of mycolic acid synthesis in BCG may occur prior to synthesis of the saturated alkyl intermediate with OSA, but between this intermediate and completed mycolates with cerulenin and thiolactomycin. Alternatively, OSA-mediated inhibition of mycolate synthesis in BCG and MAC may involve an as-yet-unidentified enzyme or enzyme system. In summary, the effects of OSA, cerulenin, and thiolactomycin are mycobacterial species specific and compound specific and inherent differences in the mycolic acid biosynthetic pathway may exist between rapid- and slow-growing mycobacteria.

ACKNOWLEDGMENTS

We thank Janet Folmer for performance of electron microscopy and William Bishai for providing equipment and laboratory space for some portions of this study.

This study was financially supported in part by NIH grant AI43846, the Raynam Research Fund, and the Becton Dickinson Centennial Fellowship in Clinical Microbiology (N.M.P.).

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